(14) Rhinitis



# Proinflammatory cytokines and eosinophil cationic protein on glandular secretion from human nasal mucosa: Regulation by corticosteroids

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**Background:** Airway hypersecretion is a common finding in rhinitis and asthma in which proinflammatory cytokines are upregulated. The effect of inflammation on glandular secretion remains unclear.

**Objective:** We sought to investigate the effect of proinflammatory cytokines and eosinophil products in in vitro lactoferrin glandular secretion from human nasal mucosa and the role of corticosteroids and IL-10 in modulating this effect.

**Methods:** Nasal explants from patients undergoing turbinectomies were incubated in a controlled atmosphere (50% $N_2$, 5% $CO_2$, and 45% $O_2$) at 37°C. Nasal explants were incubated with or without IL-1β, IL-4, IL-5, IL-8, eotaxin, GM-CSF, TNF-α, eosinophil cationic protein (ECP), IL-10, and budesonide in a time-course and dose-response fashion. Lactoferrin concentrations in culture supernatants were measured by means of ELISA.

**Results:** ECP (up to 30%) caused a dose-related stimulation of lactoferrin secretion. TNF-α (20 ng/mL) induced a significant increase of lactoferrin release from 8 hours (27.7% ± 17.8%, $P < .05$) to 24 hours (40.8% ± 17.2%, $P < .01$) compared with that found in media-treated explants. At 24 hours, TNF-α caused a dose-related stimulation of lactoferrin secretion (up to 35%). IL-1β (65.3% ± 15.2%, $P < .05$) and GM-CSF (15.7% ± 6.7%, $P < .05$), both at 20 ng/mL, exerted a stimulatory effect only at 24 hours, and IL-4, IL-5, IL-8, and eotaxin had no significant effect. Budesonide caused a dose-related inhibition of lactoferrin secretion induced by IL-1β (down to −76%) and TNF-α (down to −70%), whereas IL-10 had no effect.

**Conclusions:** ECP and some proinflammatory cytokines (IL-1β, TNF-α, and GM-CSF) may contribute to glandular hypersecretion in the inflamed nose. Corticosteroids may reduce nasal hypersecretion by blocking the direct effect of proinflammatory cytokines on glandular output. (J Allergy Clin Immunol 2001;108:87-93.)

*Key words: Nasal mucosa, glandular secretion, lactoferrin, budesonide, eosinophil cationic protein, IL-1β, TNF-α, GM-CSF, IL-10*

*Abbreviations used*
DMSO: Dimethyl sulfoxide
ECP: Eosinophil cationic protein
MPO: Myeloperoxidase
SI: Secretory index

Airway hypersecretion with mucus gland enlargement and goblet cell hyperplasia are common findings in inflammatory diseases, such as rhinitis,[1-2] asthma, and chronic obstructive pulmonary diseases.[3]

Lactoferrin is an 80-kd protein with antimicrobial properties that is released by submucosal serous cells of human upper and lower airways. Lactoferrin has been used as a serous marker of nasal gland secretion in several in vivo and in vitro studies.[4-7] Although airway glandular secretion is mainly under the control of the cholinergic system,[4] other proinflammatory mediators, such as histamine,[4] adrenergic agonists,[4] neuropeptides,[5] prostaglandins,[8] cytokines,[9-12] endothelin,[13] eicosanoids,[8] cystinyl leukotrienes,[8] platelet-activating factor,[8] bradykinin,[14] and inflammatory cell products,[15,16] may also play a role in airway gland hypersecretion.

Eosinophil infiltration is a histologic feature of rhinitic and asthmatic airways,[17,18] whereas neutrophil infiltration predominates in airways of patients with chronic obstructive pulmonary disease.[19,20] Granule proteins released by activated eosinophils and neutrophils may stimulate gland secretion.[17,18,21-23] For instance, eosinophil granule extracts and eosinophil cationic protein (ECP) induced mucus release from human bronchi and feline tracheal explants.[15] The effect of eosinophil proteins on glandular secretion from human nasal mucosa has not yet been investigated.

A variety of proinflammatory cytokines are upregulated in rhinitis (IL-1β, IL-4, IL-5, IL-6, IL-8, eotaxin, RANTES, TNF-α, and GM-CSF),[17,24-26] asthma (IL-4, IL-5, GM-CSF, TNF-α, IL-6, IL-1β, and IL-2),[3,27] and chronic obstructive pulmonary disease (TNF-α and IL-8).[28] There is little information on the role of cytokines in the regulation of human airway glandular secretion. IL-4 induced mucus production from cultured NCI-H292 epithelial cells,[29] but it had no effect on gland secretion from human nasal explants.[30] IL-1β caused a significant increase in mucus secretion,[9] whereas TNF-α induced

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mucus hypersecretion in cultured guinea pig tracheal epithelial cells[10,11] and human bronchial epithelial cells.[12]

Topical and systemic corticosteroids are widely used in the treatment of airway inflammatory diseases. Corticosteroids induce eosinophil apoptosis,[31] inhibit cytokine production from nasal mucosa,[32-34] and reverse nasal and bronchial obstruction, normalizing airway hypersecretion.[35,36] Corticosteroids reduce spontaneous and cholinergic-induced glandular secretion in human respiratory mucosa,[7] but there are no studies on the effects of corticosteroids on nasal glandular secretion stimulated by proinflammatory cytokines.

Studies conducted in human airways[17,37-40] and in transgenic mice[41] suggest that IL-10 might exert anti-inflammatory effects in the airways, but it is unknown whether IL-10 has any effect on airway gland secretion.

We hypothesize that gland hypersecretion in rhinitis is in part caused by a direct effect of proinflammatory cytokines and eosinophil products and that the inhibition of their effects may contribute to the clinical efficacy of anti-inflammatory products in reducing hypersecretion. We therefore investigated the effect of ECP and several proinflammatory cytokines (IL-1β, IL-4, IL-5, IL-8, eotaxin, GM-CSF, and TNF-α) on lactoferrin glandular secretion from human nasal explants in vitro. Finally, we studied the potential protective effects of budesonide and IL-10 in modulating this effect.

## METHODS

### Material

*Nasal explant cultures.* L-15 Leibovitz and CMRL 1066 media were obtained from Life Technologies (Paisley, Scotland, United Kingdom); budesonide and dimethyl sulfoxide (DMSO) were from Sigma-Aldrich Co (Madrid, Spain); 24-well culture plates were from Costar (Cultek SL, Madrid, Spain); and recombinant human IL-1β, IL-4, IL-5, IL-8, IL-10, eotaxin, GM-CSF, and TNF-α were from R&D Systems (Minneapolis, Minn).

*Lactoferrin ELISA.* Lactoferrin standard and *o*-phenylenediamine were obtained from Sigma-Aldrich Co; Tween-20 was from Merck (Darmstadt, Germany); rabbit anti-human lactoferrin was from Dako Diagnostics (Glostrup, Denmark); sheep polyclonal antibody anti-human lactoferrin horseradish peroxidase conjugate was from Biogenesis (Poole, United Kingdom); and microplate autoreader EL-311, ELISA autowasher EL-403, and Delta Soft II 4.0 ELISA analysis were from Bio-Tek (Winooski, Vt).

*Myeloperoxidase.* Myeloperoxidase (MPO) ELISA kits were obtained from R&D Systems.

*Isolation of ECP.* Sephadex G-50 and heparin Sepharose 6B were obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden).

### Patients

Human nasal explants were obtained from patients with nasal obstructive syndrome undergoing partial or total turbinectomies (n = 51; 28 men and 23 women; age, 33.2 ± 1.8 years). None of the patients had upper airway infections in the 2 weeks before surgery. No patient was receiving topical or systemic glucocorticoid treatment at the time of surgery. In our study 7 (14%) of the 51 nasal mucosa were obtained from inferior turbinates of atopic (positive skin test responses) patients. One patient was sensitized to mites, 4 to animal dander, and 2 to both mites and animal dander. All patients gave informed consent to participate in the study, which was approved by the scientific and ethics committee of our institution.

### Airway explant culture

In the operating room nasal specimens were placed into a 50-mL sterile tube with 20 mL of L-15 Leibovitz media supplemented with glutamine, penicillin (100 UI/mL), streptomycin (100 μg/mL), and amphotericin B (2 μg/mL) and immediately transported to the laboratory. Airway mucosal specimens were cut into 3 × 3–mm explants and placed on 24-well sterile plates (1 explant each) with 1 mL of CMRL 1066 with glutamine supplemented with penicillin (100 UI/mL), streptomycin (100 μg/mL), and amphotericin B (2 μg/mL). Plates were placed in a culture chamber under controlled atmosphere (5% $CO_2$, 45% $O_2$, and 50% $N_2$), as previously described.[4,7] The chamber was placed on a rocking platform and incubated at 37°C.

### Preparation of eosinophils

Peripheral blood eosinophils were obtained from 4 patients with idiopathic hypereosinophilia. To obtain eosinophil granules, in which the collection of a large number of eosinophils was required, eosinopheresis was performed as previously described.[15] After eosinopheresis, the granule-enriched supernatant was centrifuged (10,000*g*), and pellets with eosinophil granules were sonicated and applied to an affinity chromatography column to obtain ECP and eosinophil-derived neurotoxin protein fractions. Protein concentrations were measured by means of absorbance spectroscopy at 277 nm.

### Lactoferrin ELISA

Lactoferrin was measured by using a modified noncompetitive ELISA, as previously described.[4,7] Briefly, 50 μL of rabbit polyclonal antibody to human lactoferrin (1:1000) was added to microtiter plates and incubated at 37°C for 90 minutes. Nonspecific binding was blocked with 1% goat serum diluted in washing buffer. After washing, lactoferrin standards or samples (50 μL) were added for 90 minutes at 37°C. Plates were washed, and sheep polyclonal horseradish peroxidase–conjugated antibody to human lactoferrin (1:2000) was added and incubated at 37°C for 90 minutes. The reaction was developed with *o*-phenylenediamine dihydrochloride and stopped with 2N HCl. Optical densities were measured by using a reference wavelength (490 nm) and a test wavelength (405 nm). Lactoferrin concentrations in the supernatants were interpolated from the standard curve by using the Delta Soft II 4.0 software package. Lactoferrin assay range was between 1.5 and 200 ng/mL. None of the selected drugs showed any effect on the final ELISA values.

### MPO ELISA

To ascertain whether lactoferrin is of glandular origin, neutrophil origin, or both, we assayed the supernatant levels of MPO by using a sandwich ELISA, as previously described.[7] Antigen captured with a solid-phase mAb was detected with a biotin-labeled goat polyclonal anti-MPO. An avidin alkaline phosphatase conjugate was then bound to the biotinylated antibody. The alkaline phosphatase substracte *p*-nitrophenyl phosphate was added, and the yellow product was read at 405 nm. The range of the MPO assay was between 1.6 and 100 ng/mL. None of the selected drugs showed any effect on the final ELISA values.

### Experimental design

Explants were incubated with culture media over a 48-hour stabilization period, and culture medium was changed every day. Every 3 to 4 wells containing the airway explants constituted the control group (media-treated explants) or the experimental group (drug-treated explants). Because budesonide was diluted in DMSO, we investigated the effect of DMSO on lactoferrin secretion. The final concentration of DMSO (0.01%) had no effect on lactoferrin secretion (data not shown).[7] Experimental designs for specific

experiments are further described in the result section. Explant supernatants were frozen at –80°C until assayed.

### Statistical analysis

Secretory indexes (SIs) were calculated as previously described to standardize lactoferrin secretion from different tissue samples containing different relative numbers of secretory cells and day-to-day variations in glandular secretion.[7] The use of SIs reduced the intraexperiment and interexperiment variations. For example, $SI_{1-2}$ was the ratio of lactoferrin from period 2 to the lactoferrin from period 1 for one group of identically treated plates on 1 day. $SI_{1-3}$ was the ratio of lactoferrin from period 3 to the lactoferrin from period 1. The $SI_{1-2}$ and $SI_{1-3}$ for samples treated in an identical fashion on a single day were determined and averaged, and the percentage change from the negative control group was determined. These results were expressed as mean ± SEM of percentage change from control. Statistical analyses were performed by using ANOVA with the Dunnett *t* test comparison in time-course experiments and a nonparametric test (Wilcoxon signed-rank test) in dose-response experiments. A *P* value of less than .05 was considered statistically significant.

## RESULTS

### Effect of ECP on lactoferrin release from human nasal mucosa

Explants were incubated with ECP (2.5-25 μg/mL) for 1 hour (Fig 1). Only ECP at 25 μg/mL caused a significant increase on lactoferrin secretion (30% ± 3.5% change from control values, $P < .05$, n = 3).

### Effect of cytokines on lactoferrin release from nasal mucosa

*Time course.* Nasal explants were incubated with selected cytokines at 20 ng/mL for 1, 8, and 24 hours. TNF-α induced a significant release of lactoferrin at 8 hours (27.7% ± 17.8% change from control values, $P < .05$, n = 7) and 24 hours (40.8% ± 17.2%, $P < .01$, n = 7; Fig 2). IL-1β (65.3% ± 15.2%, $P < .01$, n = 7) and GM-CSF (15.7% ± 6.7%, $P < .05$, n = 7) had a stimulatory effect on lactoferrin secretion only at 24 hours (Fig 3 and Table I, respectively). Eotaxin, IL-4, IL-5, and IL-8 caused no significant changes on lactoferrin secretion (Table I).

*Dose response.* Nasal explants were incubated with IL-1β, TNF-α, or GM-CSF at different concentrations (0.2, 2, and 20 ng/mL) for 24 hours. TNF-α and IL-1β caused a dose-related stimulation of lactoferrin secretion at 2 ng/mL (TNF-α: 31.6% ± 11.3%, $P < .05$, n = 12; IL-1β: 12.7% ± 7.6%, $P < .05$, n = 9) and 20 ng/mL (TNF-α: 35.5% ± 13.8%, $P < .01$, n = 19; IL-1β: 66.7% ± 18.2%, $P < .01$, n = 21; Figs 2 and 3, respectively). GM-CSF caused no significant changes in lactoferrin secretion at lower concentrations than 20 ng/mL.

### Effect of budesonide on cytokine-induced lactoferrin release from nasal mucosa

Explants were incubated with TNF-α or IL-1β (at 20 ng/mL) with or without budesonide ($10^{-10}$ to $10^{-6}$ mol/L) for 24 hours (Fig 4). The induction of lactoferrin secretion by either TNF-α or IL-1β was used as a positive control (100%) to which the inhibitory effect of budesonide was

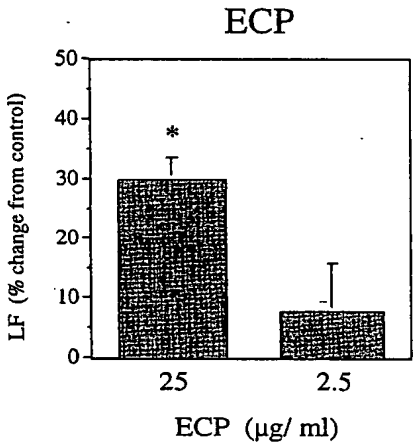


**FIG 1.** Effect of ECP on lactoferrin *(LF)* release from human nasal mucosa. Explants were incubated with ECP (2.5-25 μg/mL) for 1 hour. ECP caused a dose-related increase on lactoferrin secretion compared with that of controls. **P* < .05, Wilcoxon signed-rank test.

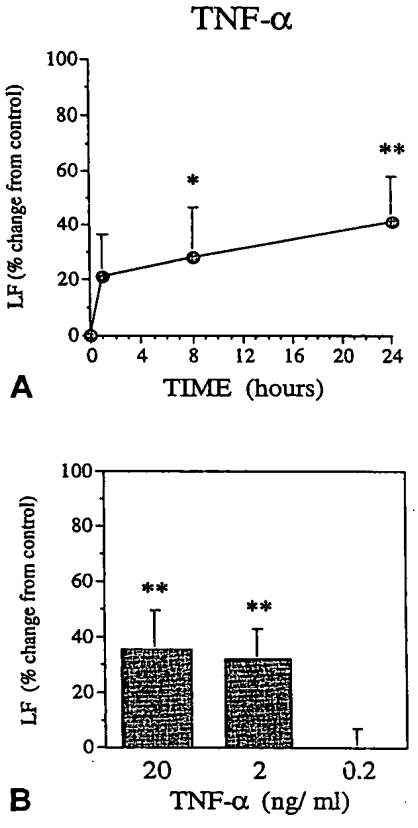


**FIG 2.** Effect of TNF-α on lactoferrin *(LF)* release from human nasal mucosa. **A,** *Upper panel,* Nasal explants were incubated with TNF-α (20 ng/mL) for 1, 8, and 24 hours. TNF-α induced a significant lactoferrin release at 8 and 24 hours compared with media-treated explants. **P* < .05, ***P* < .01, ANOVA with Dunnett *t* test comparison. **B,** *Lower panel,* Explants were incubated with TNF-α (0.2-20 ng/mL) for 24 hours. TNF-α significantly increased lactoferrin release in a dose-related fashion. ***P* < .01, Wilcoxon signed-rank test.

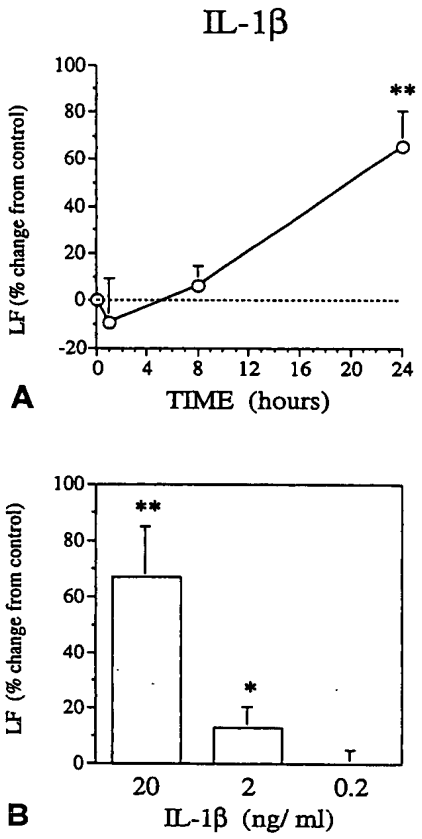


**FIG 3.** Effect of IL-1β on lactoferrin *(LF)* release from human nasal mucosa. **A**, *Upper panel*, Nasal explants were incubated with IL-1β (20 ng/mL) for 1, 8, and 24 hours. IL-1β induced a significant release of lactoferrin at 24 hours compared with media-treated explants. $^{**}P < .01$, ANOVA with Dunnett *t* test comparison. **B**, *Lower panel*, Explants were incubated with IL-1β (0.2-20 ng/mL) for 24 hours. IL-1β caused a dose-related enhancement in lactoferrin release. $^{*}P < .05$, $^{**}P < .01$, Wilcoxon signed-rank test.

**TABLE I.** Effect of cytokines on lactoferrin secretion from nasal mucosa explants

| | Lactoferrin (% change from control) | | |
|---|---|---|---|
| **Cytokines (20 ng/mL)** | **1 h** | **8 h** | **24 h** |
| Eotaxin (n = 4) | 11.4 ± 31.4 | 7.3 ± 12.4 | −0.6 ± 7.9 |
| GM-CSF (n = 7) | −20.2 ± 22.2 | −7.2 ± 10.7 | 15.7 ± 6.7* |
| IL-4 (n = 4) | −24.3 ± 29.9 | 11.4 ± 26.5 | −10.8 ± 13.1 |
| IL-5 (n = 4) | −16.3 ± 23.7 | 18.2 ± 12.8 | 7.9 ± 4.0 |
| IL-8 (n = 7) | 6.6 ± 27.4 | 6.9 ± 15.2 | −2.8 ± 4.8 |

*$P < .05$, Wilcoxon signed-rank test.

compared. Budesonide (n = 7) caused a dose-related inhibitory effect on lactoferrin secretion induced by both IL-1β ($10^{-10}$ mol/L: −56.1% ± 23.1%, $P < .05$; $10^{-8}$ mol/L: −73.4% ± 44.7%, $P < .05$; $10^{-6}$ mol/L: −76.3% ± 13.6%, $P < .01$) and TNF-α ($10^{-6}$ mol/L: −70.3% ± 28.5%, $P < .05$).

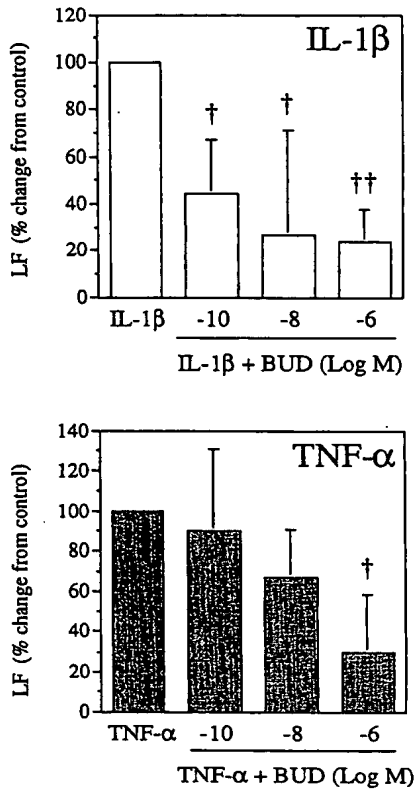


**FIG 4.** Effect of budesonide on cytokine-induced lactoferrin *(LF)* release from human nasal mucosa. Explants were incubated with IL-1β or TNF-α (20 ng/mL) in the presence or absence of budesonide ($10^{-10}$ to $10^{-6}$ mol/L) for 24 hours. Budesonide caused a dose-related inhibition of the lactoferrin release induced by both IL-1β and TNF-α compared with cytokine-treated explants. $^{\dagger}P < .05$, $^{\dagger\dagger}P < .01$, Wilcoxon signed-rank test.

## Effect of IL-10 on IL-1β–induced lactoferrin release from nasal mucosa

Explants were incubated with IL-1β at 20 ng/mL in the presence or absence of IL-10 (20 ng/mL) for 24 hours (n = 5). Induction of lactoferrin secretion by IL-1β was used as a positive control (100%) to which the effect of IL-10 was compared. IL-10 did not show any effect on either basal (6.4% ± 12.4% compared with spontaneous secretion) or IL-1β–induced lactoferrin secretion (−21.6% ± 25% change from control).

## MPO

Low amounts of MPO were found in the explant supernatants, and no significant differences were observed in supernatant MPO concentrations between media-treated (10.8 ± 4.2 ng/mL, n = 9) and cytokine-treated tissues (TNF-α: 5.9 ± 2.7 ng/mL, n = 8; IL-1β: 10.1 ± 4.0 ng/mL, n = 5; GM-CSF: 11.4 ± 6.0 ng/mL, n = 6), suggesting that lactoferrin measured in cultured nasal explants was originated in glandular cells.

## DISCUSSION

The main findings of our study on in vitro human nasal glandular secretion are as follows: (1) ECP caused an increase in lactoferrin secretion; (2) IL-1β, TNF-α, and, to a lesser extent, GM-CSF induced lactoferrin secretion while IL-4, IL-5, IL-8, and eotaxin showed no effect; and (3) budesonide inhibited lactoferrin secretion induced by both IL-1β and TNF-α, whereas IL-10 had no effect. These results suggest that some proinflammatory cytokines along with ECP may play a role in the regulation of nasal hypersecretion.

Eosinophil infiltration of the nasal mucosa has been reported as a histologic hallmark of nasal inflammatory diseases, such as allergic rhinitis,[42] allergic sinusitis,[43] and nasal polyposis.[44] ECP is a protein released by activated eosinophils with an important role in nasal mucosa inflammation.[17,45] Lundgren et al[15] reported that eosinophil granule extracts stimulated in vitro mucus secretion from feline trachea, and ECP stimulated glandular secretion from both cultured human bronchi and feline trachea. In keeping with these studies, our results suggest that ECP exerts a direct stimulatory effect on nasal gland secretion. Previous studies have also shown that ECP at the concentrations used has no cytotoxic effect on the airways.[15,46]

Proinflammatory cytokines have proved to be upregulated in airway inflammatory diseases, such as asthma,[27] chronic obstructive pulmonary diseases,[28] and rhinitis. For instance, concentrations of IL-1β, IL-4, and IL-5 are increased in nasal secretions from patients with allergic rhinitis.[17,42,47] By using various detection techniques, the expression and secretion of eotaxin, IL-1β, IL-8, GM-CSF, and TNF-α, as well as those of IL-1 and TNF-α receptors, have also proved to be upregulated in nasal epithelial cells from patients with rhinitis when compared with healthy volunteers.[24,26,48]

In our in vitro model, IL-1β, TNF-α, and GM-CSF had a stimulatory effect on nasal glandular secretion, whereas IL-4, IL-5, IL-8, and eotaxin had no effect. TNF-α had an earlier effect (from 8 hours) than IL-1β and GM-CSF (at 24 hours). These findings are in keeping with previous studies carried out in the lower airway, showing that proinflammatory cytokines may increase glandular secretion.[9,11,12] TNF-α induced mucin hypersecretion from in vitro guinea pig tracheal epithelial cells,[11] as well as from cultured human bronchial explants and bronchial epithelial cells.[12] Significant increases in mucin secretion from cultured human bronchi were also detected after stimulation with IL-1β.[9] In our study IL-4 had no effect on glandular secretion, confirming previous studies that, by using similar concentrations of IL-4 (25 ng/mL) but different incubation times (1, 6, and 12 hours), were unable to demonstrate any effect on in vitro nasal gland secretion.[30] Therefore these cytokines may play a role in the hypersecretion usually observed in upper airway diseases characterized by an increased expression of cytokines.

Corticosteroids downregulate nasal mucosa inflammation either by inducing anti-inflammatory factors[35] or cell apoptosis[32,49] or by inhibiting the generation of proinflammatory cytokines.[25,32-34,36] The effect of corticosteroids on upper and lower airway hypersecretion has been a matter of debate. Studies in in vitro human and feline lower airways have reported that glucocorticoids have inhibitory effects on mucus secretion.[50-52] In contrast, Ali et al[6] reported no effects of dexamethasone on nasal gland secretion in vitro. Moreover, McGregor et al[53] showed that fluticasone propionate and beclomethasone dipropionate appear to enhance mucin secretion in the normal nose. We have recently reported the inhibitory effect of corticosteroids on spontaneous and methacholine-induced glandular secretion in human nasal and bronchial mucosa in vitro.[7] In the present study we have found that corticosteroids were capable of reducing the stimulatory effect of some cytokines (TNF-α and IL-1β) on gland output. In a previous study we reported that corticosteroids have no cytotoxic effect on cultured nasal mucosa.[7] Therefore the reduction in hypersecretion attained with corticosteroids in the inflamed nasal mucosa appears to be due, at least in part, to the ability of these drugs to abrogate the stimulatory effects of some cytokines.

Studies conducted in airway mucosa have suggested the potential role of IL-10 in the pathogenesis of airway inflammation. For instance, the expression of IL-10 in human nasal[54] and bronchial[55] mucosa is increased in response to allergen exposure. IL-10 strongly inhibits the cytokine-induced cyclooxygenase 2 expression in the nasal mucosa.[40] Finally, Yang et al[41] have reported a significant reduction of mucus production in IL-10 knockout mice. However, in our model IL-10 had no effect on either basal or IL-1β–induced lactoferrin secretion. This result suggests that IL-10 does not participate in the regulation of glandular secretion.

In summary, the inflammatory response arising in the airway of patients with rhinitis, asthma, and chronic obstructive pulmonary diseases is usually accompanied by hypersecretion. Hypersecretion is probably caused by the combined action of numerous mediators released during the inflammatory response, including proinflammatory cytokines. Our study suggests that the local generation of some cytokines, such as TNF-α, IL-1β, and GM-CSF, as well as ECP, may stimulate the output of secretory cells. On the other hand, topical corticosteroids (budesonide) may reduce nasal hypersecretion by blocking the effect of cytokines on glandular secretion.

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